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Regulation of Hematopoietic Growth Factor Production by Genetically Modified Human Bone Marrow Stromal Cells Expressing Interleukin-1β Antisense RNA

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ABSTRACT

Interleukin-1 (IL-1) plays a major role in the regulation of bone marrow stromal cell function and hematopoiesis. It is known to induce secretion of the hematopoietic growth factors granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), IL-6, and IL-8 as well as IL-1 itself in stromal cells. We investigated the role of IL-1β-mediated growth factor production in the human stromal cell line L88/5. Using liposome-mediated DNA transfer, two stromal cell transfectants that constitutively express IL-1β antisense (AS) RNA were generated. Expression of IL-1β AS RNA and IL-1β RNA was determined by RT-PCR. The stromal cell transfectants were strongly impaired in their endogenous IL-1β production, and this effect was present even when strong IL-1α inducers, such as IL-1α and tumor necrosis factor-α (TNF-α), were used. Reduced endogenous IL-1β levels had no effect on the constitutive production of IL-6, IL-8, and GM-CSF measured by ELISA. In contrast to lipopolysaccharide (LPS) stimulation, IL-1α-mediated stimulation of GM-CSF production was significantly reduced in AS transfectants. TNF-α induced GM-CSF production was also reduced. IL-6 and IL-8 production was increased in transfectants, suggesting a negative regulatory role of IL-1β in L88/5. This new approach using AS technology to specifically target constitutive RNA expression will allow further characterization of the bone marrow cytokine network in normal and malignant hematopoiesis.

INTRODUCTION

INTERLEUKIN-1β (IL-1β), A MEMBER OF THE IL-1 gene family, is produced by a variety of cells in response to inflammatory stimuli. IL-1β and IL-1α function as agonists on IL-1 receptors, whereas IL-1 receptor antagonist (IL-1RA) is a specific receptor antagonist. Biologic activities of IL-1 affect nearly every cell type, often in combination with other cytokines or mediator molecules. In contrast to IL-1α, which seems to be primarily a regulator of intracellular events, IL-1β is a systemic, hormonelike mediator molecule.[1] Regulation of IL-1β production and activity seems to be highly complex, and several regulating factors have been identified.[1,2,3] In hematopoiesis, IL-1β is a central cytokine regulating the expression of growth factors and adhesion molecules by bone marrow stromal cells and accessory bone marrow cells. In human long-term bone marrow culture, IL-1β acts synergistically with several colony-stimulating factors (CSF) on proliferation of myeloid progenitors cells.[4] This synergism with CSF is most apparent in *ex vivo* expansion of enriched hematopoietic progenitor cells.[5] Indirect actions on hematopoiesis include the induction of hematopoietic growth factors, such as IL-1β, IL-3, granulocyte-macrophage CSF (GM-CSF), and granulocyte CSF (G-CSF).[6–11] IL-1α and tumor necrosis factor-α (TNF-α) have been reported to act synergistically to stimulate GM-CSF and G-CSF production in primary human bone marrow stromal cells and clonally derived stromal cell lines, thus affecting hematopoiesis.[12,13] Another mechanism of IL-1 action in hematopoiesis is exerted through the upregulation of CSF receptors and adhesion molecules, as shown for c-kit[14] and intracellular adhesion molecule-1 (ICAM-1).[15] Interestingly, IL-1β itself seems not to be essential for normal stem cell proliferation or differentiation. Data from IL-1β-deficient mice, which show no hematologic impairment when healthy, support this conclusion.[16]

Regulation of differentiation and proliferation of hemato-

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poietic progenitor cells is a highly complex process. Main cellular elements of the bone marrow microenvironment regulating hematopoiesis in health and in disease include stromal cells, accessory bone marrow cells, such as macrophages, fibroblasts, adipocytes, endothelial cells, and the hematopoietic cells itself. Soluble factors and extracellular matrix components produced by these cells maintain the functional integrity of this microenvironment.[17,18] The establishment of long-term bone marrow culture systems using stromal cell monolayers has allowed investigation of bone marrow regulatory processes *in vitro*.[19]

Although a number of spontaneously immortalized murine stromal cell lines have been reported,[20, 21] attempts to generate human bone marrow stromal cell lines merely by serial passaging have failed. Simian virus 40 (SV40) transformation has allowed the establishment of permanent murine[22] and human stromal cell lines.[23] SV40-transformed L88/5 stromal cells have been shown to support the proliferation of $CD34^+$ human cord blood cells, with a maximum of colony-forming cell (CFC) production after 3 weeks.[24,25]

The aim of the present study was to examine the role of stromal cell-derived IL-1β on the expression of endogenous growth factors in stromal cells under various stimulatory conditions. We generated two human bone marrow stromal cell transfectants deficient for expression of endogenous IL-1β by constitutive IL-1β antisense (AS) RNA expression using the L88/5 stromal cell line. Cytokine production by stromal cells expressing the IL-1 AS construct was investigated under various stimulatory conditions. Our results suggest a differential regulatory mechanism of IL-1β on the endogenous expression of various growth factors in stromal cells. This new approach will allow characterization of IL-1β dependent cytokine regulation in normal and malignant human hematopoiesis.

MATERIALS AND METHODS

Cell culture

The human bone marrow stromal cell line L88/5[24] (kindly provided by P. Dörmer) was used in all studies. Cells were cultured in McCoy's 5A medium (Biochrom, Berlin, Germany) supplemented with 5 mmol/L L-glutamine, 0.1 mM β-mercaptoethanol (β-ME), 1% Antibiotic-Antimycotic® (GIBCO-BRL, Inchinnan, U.K.) solution consisting of 10,000 IU penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B, 1% sodium bicarbonate, 1 mmol/L sodium pyruvate, 0.8% nonessential amino acids, 0.1 mmol/L monothioglycerol, 0.01 mmol/L hydrocortisone, 12.5% fetal bovine serum (FBS), and 12.5% horse serum (Greiner, Frickenhofen, Germany) and maintained in flasks. At medium renewal, cells grown to confluent layers were detached with phosphate-buffered saline (PBS) containing 0.05% trypsin/0.02% EDTA (Biochrom) and recultured.

Growth factors, reagents, and antibodies

Recombinant human IL-1α (rHuIL-1α), rHuIL-1β, and rHuTNF-α were purchased from Genzyme (Cambridge, MA). Lipopolysaccharide (LPS) (*Escherichia coli* S027: B8) was obtained from Sigma (Deisenhofen, Germany). Monoclonal antibodies (mAb) to HuIL-1β, HuIL-6 and Hu IL-8 were purchased from R & D Systems (Minneapolis, MN). mAb to HuGM-CSF were obtained as a complete Biotrak® ELISA system from Amersham Life Science (Little Chalfont, U.K.). All molecular biology reagents used for cloning were supplied by Roche Diagnostics (Mannheim, Germany). Geneticinin sulfate (G418) was obtained from Calbiochem (La Jolla, CA).

IL-1β AS oligonucleotides and RNase H digestion

The following IL-1β AS oligonucleotides were designed on the basis of a nonhomology search comparing the HuIL-1β gene[26] with other available sequences of the human genome: HuIL-1β AS1: 5'-CCATGGCTGCTTCAGACA-3', HuIL-1β AS2: 5'-TTGCTCCATATCCTGTCC-3', HuIL-1β AS3: 5'-GCTTGAGAGGTGCTGATG-3'. Oligonucleotides nonspecific for HuIL-1β (Nonsense: 5'-GCTGACCATCAATAAG-GAAG-3') or specific for HuIL-1β in sense orientation (Sense:5'-GACCTGGACCTCTGCCCTCTGG-3') were employed as controls. Total mRNA from the promonocytic cell line HL-60 stimulated overnight with 10 ng/ml phorbol myristate acetate (PMA) (Sigma) was used to test for specific and efficient binding of the oligonucleotides to HuIL-1β RNA. Each oligonucleotide was incubated with total RNA, followed by digestion with RNase H and transcription of mRNA into first-strand IL-1β cDNA by an IL-1β-specific downstream primer. The amount of IL-1β cDNA obtained after RNase H treatment was assessed by semiquantative RT-PCR using IL-1β-specific primers (IL-1β 5': GGCTGCTCTGGGATTCTCTT; IL-1β 3': AGTGAGTAGGAGAGGTGAGAGAGGCCTGG) and β-actin RT-PCR product to ensure comparable amounts of cDNA used as template.

IL-1β RT-PCR and preparation of IL-1β AS constructs

Total RNA was extracted from the HL-60 cell line using the RNAzol B Isolation method.[27] Briefly, adherent monolayers of HL-60 cells stimulated 10 ng/ml PMA for 6 h were lysed directly using guanidine thiocyanate/phenol mixture, followed by chloroform extraction and precipitation in isopropanol with glycogen. The RNA pellet was washed in 75% ethanol and RNA solubilized in water prepared RNase free by diethylpyrocarbonate (DEPC) treatment.

First-strand synthesis was performed by mixing 2 μg cellular RNA, 5 μmol/L random hexamer oligonucleotides (Perkin-Elmer/Roche, Branchburg, NJ), 0.6 mmol/L dNTP mix, and 10 mmol/L dithiothreitol (DTT) with buffer (50 mmol/L) KCl, 10 mmol Tris-Cl, pH 8.3, and 5 mmol/L $MgCl_2$ in a volume of 20 μl), heating to 95°C for 5 min, cooling on ice, and then adding 10μm RNasin (Perkin-Elmer/Roche) and 20μm murine leukemia virus (MuLV) (Perkin-Elmer/Roche) to a total volume of 22 μl. The mixture was first incubated for 10 min at ambient temperature, followed by 40°C for 40 min, and finished after 10 min at ambient temperature. After first-strand synthesis, HuIL-1β was amplified with 50 pmol/L of each primer and 1.25μ Amplitaq DNA polymerase (Perkin-Elmer/Roche), using up to 10 μl cDNA mix. The IL-1β sense primer 5'-TTTGAATTCTCATTGCTCAAGTGTCTGAAGCAGC-CATGG-3' and IL-1β AS primer 5'-TTTGGATCCTGGAAG-GAGCACTTCATCTGTTTA-3' were used to generate a 136-bp fragment, whereas the IL-1β sense primer in combination with the IL-β AS primer 5'-TTTGGATCCAGAAGATAGGT-

TCTTCTTCAAAGAT-3′ amplified a 402-bp fragment. Both primers were modified to include an EcoRI restriction site at the 5′-end and a BamHI site at the 3′-end. As an internal control, β-actin RNA was subjected to RT-PCR using β-actin-specific primers (sense primer: 5′-TCCTGTGGCATCCAC-GAAACT-3′; AS primer: 5′-GAAGCATTTGCGGTGGAC-GAT-3′). Amplification was carried out for 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a 10-min extension after the last cycle in an Omni-Gene Thermal Cycler (Hybaid, Teddington, U.K.) in 200 μmoL/l dNTP mix and Perkin-Elmer Taq buffer. PCR products were visualized by UV illumination of ethidium bromide-stained gels, followed by excision of bands with correct size and isolation of DNA using a Prep-Gene DNA purification system (Bio-Rad, Hercules, CA).

The correct sequence of the PCR products of 136 bp and 402 bp in length was verified by sequencing, and each IL-1β insert was cloned as BamHI/EcoRI fragments in reverse orientation into the pcDNA 3.1 (Invitrogen, Leek, The Netherlands) derivative expression vector pCMVEx. A random sequence of 106 bp was cloned into the vector using HindIII/ApaI restriction sites and used as a mock (nonsense) control.

Generation and PCR analysis of stable transfectants

L88/5 cells were cotransfected with AS construct carrying either the 136-bp IL-1β insert designated pCMVExHuIL-1βAS1 or the 402-bp insert designated pCMVExHuIL-1βAS2 and the pSV2neo vector conferring neomycin resistance by cationic liposome-mediated DNA transfer using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP) (Roche Diagnostics). Stable transfectants were established in the presence of G418 (final concentration 500 μg/ml).

For genomic PCR analysis of IL-1β AS transfectants, extracts from $1.5–2 \times 10^4$ cells were prepared by lysis (10 min at 95°C in 20 μl water) and proteinase K treatment (400 μg/ml) for 60 min at 55°C, followed by heat inactivation for 10 min at 95°C, and subjected to hot start PCR using the pcDNA 3.1

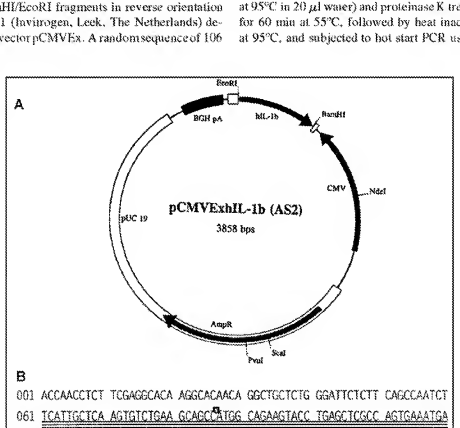

FIG. 1. (**A**) The expression construct for constitutive expression of IL-1β AS RNA of 402 bp: pCMVExhIL-1β (AS2). One IL-1β fragment of 402 bp in length encompassing the translation initiation codon (ATG) and exon/intron splicing sites for the first 2 exons was generated by PCR and designated HuIL-1AS2 (402 bp). The fragment was cloned in AS orientation into a pcDNA 3 derivative eukaryotic expression vector using the EcoRI and BamHI sites. Expression of the IL-1β sequence is driven by the CMV promoter (CMV) and tailed with a poly A tail from the bovine growth hormone gene (BGH). Further components of this vector consist of a T7 and a SP6 polymerase initiation site, respectively, and the β lactamase gene conferring ampicillin resistance (AmpR). (**B**) The human IL-1β sequence generated by PCR to be cloned in AS orientation. IL-1βAS1 (136bp): triple underlined; IL-1βAS2 (402 bp), solid underlined; arrows, exon/intron splicing sites.

vector-specific sense primer 5′-ACGACTCACTATAGGGA-GACC-3′ and the appropriate IL-1β-specific AS primer (for the 136-bp fragment of IL-1β: 5′-TGGAAGGAGCACTTCA-TCTGTTTA-3′, and for the 402-bp fragment of IL-1β: 5′-AGAAGATAGGTTCTTCTTCAAAGAT-3′). As a specificity control to verify correct size and expression of IL-1β AS RNA, one sample of each transfectant was subjected to hot start PCR in the presence of 10 ng pCMVExIL-1βAS plasmid. In addition, β-actin RNA or RNA of the neomycin resistance gene was subjected to RT-PCR using β-actin-specific primers as described or specific primers to the neomycin resistance gene. Amplification was performed for 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, with a 10-min extension after the last cycle. PCR products were fractionated by electrophoresis in 1% agarose gels, followed by Southern blot analysis using ^{32}P-end-labeled hybridization primers specific for the 136-bp fragment and 402-bp fragment of IL-1β or for β-actin.

Cytokine immunoassays

The presence of GM-CSF was evaluated using a Biotrak® HuGM-CSF system (Amersham Life Sciences) as recommended by the manufacturer. The cytokines IL-1β, IL-6, and IL-8 were measured by ELISA. Briefly, microtiter wells were coated (4 μg/ml) with anti-IL-1β (clone 2805.31), anti-IL-6 (clone 6708.11), or anti-IL-8 (clone 6217.11) capture antibodies. Samples to be tested were added sequentially, followed by the appropriate biotinylated second anti-IL-1β (100 ng/ml), anti-IL-6 (25 ng/ml), or anti-IL-8 (20 ng/ml) mAb directed against different epitopes on the cytokine molecules and avidin-conjugated horseradish peroxidase (HRP). Bound cytokine molecules were revealed colorimetrically after the addition of peroxidase substrate. The sensitivity of the assays has been found to be in the order of 1–3 pg/ml.

In the case of IL-1β-containing samples, membrane-bound IL-1β was determined. Adherent cell layers were detached by incubating the flask for 5 min at 37°C with PBS containing 0.05% trypsin/0.02% EDTA. Cells were sedimented and sonicated, and the extract was reconstituted in 1 ml PBS to be used without any further biochemical purification for the IL-1β immunoassay.

RESULTS

Generation of human bone marrow stromal cell transfectants expressing HuIL-1β AS RNA

To study the role of IL-1β on the regulation of production of such hematopoietic growth factors as GM-CSF, IL-6, and IL-8 by stromal cells, we established human bone marrow cell transfectants that constitutively express HuIL-1β AS RNA. In order to define sequences within the HuIL-1β mRNA that did not show homology to any human sequences currently known and could, therefore, be targeted efficiently by AS RNA molecules, a BLASTN alignment search of the IL-1β gene with available sequences of the human genome was conducted. Three sequence regions within the IL-1β mRNA showing no or low homology to other human sequences were defined and further examined for specific and efficient binding by IL-1β AS oligonucleotides derived from sequences of those three non-homologous regions. The binding of AS oligonucleotides to IL-1β RNA on incubation with total RNA from activated HL-60 cells expressing high levels of IL-1β RNA led to the formation of various degrees of DNA-RNA hybrids. Treatment with RNase H, known to be activated by DNA-RNA hybrids,[28] resulted in specific cleavage of the IL-1β AS oligonucleotide-HuIL-1β RNA hybrids as assessed by amplification of the remaining IL-1β RNA using semiquantitative RT-PCR, with comparable amounts of β-actin RNA as control (data not shown). The results obtained from these studies indicated that IL-1β AS oligonucleotides specific for IL-1β sequences encompassed by exon 1–exon 4 could sufficiently induce cleavage of HuIL-1β RNA by RNAse H compared with an IL-1β AS oligonucleotide binding to a sequence within region 3 and a sense oligonucleotide used as control.

Therefore, two IL-1β fragments of 136 bp and 402 bp in length encompassing the translation initiation codon (ATG) and exon/intron splicing sites for the first 3 exons of the IL-1β gene, respectively, were generated by RT-PCR and designated HuIL-1βAS1 (136 bp) and HuIL-1βAS2 (402 bp). Different constructs, each composed of an IL-1β fragment or a 106-bp nonsense sequence (NS) cloned under the control of a CMV

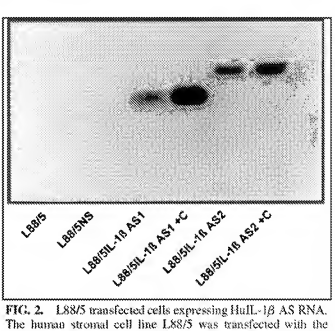

FIG. 2. L88/5 transfected cells expressing HuIL-1β AS RNA. The human stromal cell line L88/5 was transfected with the CMVEXHuIL-1AS1 and CMVEXAS2 constructs, respectively, and cotransfected with the pSV2neo plasmid to confer neomycin resistance. As an internal control, L88/5 cells were also transfected with CMVEX containing a nonsense sequence of 106 bp (CMVEXNS). Transfectants were selected in the presence of G418 and screened for stable integration of HuIL-1 AS DNA by genomic PCR using vector-specific and HuIL-1β antisense-specific primers. L88/5, parental cell line; L88/5 NS, L88/5 cells transfected with a vector containing a nonspecific (nonsense) control DNA sequence; L88/5IL-1βAS1, L88/5 cells transfected with 136-bp HuIL-1β AS DNA fragment; L88/5IL-1βAS1 + C, L88/5 AS1 transfectants subjected to PCR in the presence of 10 ng CMVEXHuIL-1AS plasmid as control; L88/5IL-1βAS2, same as L88/5IL-1βAS1 but transfected with 402-bp HuIL-1βAS DNA fragment; L88/5IL-1βAS2 + C, L88/5IL-1β AS2 transfectants subjected to PCR in the presence of 10 ng CMVEX-HuIL-1βAS plasmid as control.

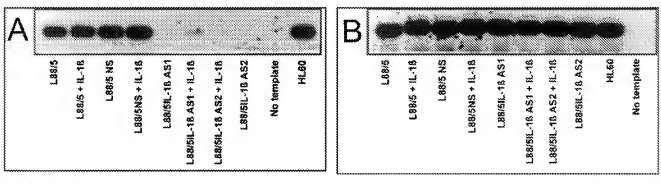

FIG. 3. (**A**) Expression of HuIL-1β RNA in L88/5 HuIL-1β AS transfectants. Expression of endogenous IL-1β RNA in the L88/5 transfectants was tested by semiquantitative RT-PCR. Total RNA was extracted from cells cultured either in medium or in the presence of 200 U IL-1β/ml for 8 h, followed by cDNA synthesis and PCR using IL-1β primer specific for amplification of IL-1β RNA in the presence of IL-1β AS RNA. L88/5 HuIL-1β AS transfectants expressed much lower levels of IL-1β RNA compared with the L88/5 NS control or the L88/5 parental cells. Total RNA from the promonocytic HL-60 cell line (which had been stimulated with 10 ng PMA/ml for 6 h) and amplification performed without template were used as positive and negative controls, respectively. (**B**) Amplification of β-actin. To be able to compare the different amounts of IL-1β RNA PCR product, β-actin RNA was amplified. L88/5, parental cell line; L88/5 NS: L88/5 cells transfected with a vector containing a nonspecific (nonsense) control DNA sequence; L88/5IL-1βAS1, L88/5 cells transfected with 136-bp HuIL-1β AS DNA fragment; L88/5IL-1βAS2, L88/5 cells transfected with 402-bp HuIL-1β AS DNA fragment; No template, PCR control without DNA; HL60, HL-60 cells; L88/5 + IL-1β, L88/5 NS + IL-1β; L88/5IL-1βAS1 + IL-1β, L88/5IL-1βAS2 + IL-1β, parental L88/5 cells or transfectants stimulated with 200 U/ml IL-1β for 8 h.

promoter in antisense orientation into a pcDNA 3 derivative eukaryotic expression vector using BamHI/EcoRI restriction sites or HindIII/ApaI sites for the NS mock control were prepared and designated pCMVExHuIL-1βAS1 (AS1), pCMVExHuIL-1βAS2 (AS2), and pCMVExNS (NS), respectively (Fig. 1). Stable cell transfectants of the permanent human bone marrow stromal cell line L88/5[241] cotransfected with the AS constructs and pSV2Neo plasmid conferring G418 resistance were selected in the presence of G418, and subcloned lines were screened for stable integration of HuIL-1β AS DNA by genomic PCR using vector-specific and IL-1β AS-specific primers that could not amplify endogenous IL-1β.

The transfected cell lines L88/5AS1 and L88/5AS2 showed stable expression of HuIL-1β AS DNA (Fig. 2) as well as nonsense DNA (not shown) to comparable levels. The presence of IL-1β AS DNA was checked in fixed periods of time while maintaining the cells in culture by genomic PCR using the appropriate pCMVExHuIL-1βAS1 or pCMVExHuIL-1βAS2 plasmid DNA exogenously added to transfected cells as positive control (e.g., depicted in Fig. 2 as L88/5IL-1β AS1 + C).

Reduced expression of endogenous IL-1β in HuIL-1β AS RNA transfectants

The expression of HuIL-1β in the L88/5 transfectants was investigated at the RNA level. We assessed the amount of endogenous HuIL-1β RNA expressed in the transfected cells by semiquantitative RT-PCR using β-actin RNA levels to compare different amounts of IL-1β PCR product. Total RNA was extracted from L88/5 cells cultured either in medium alone or in the presence of exogenously added HuIL-1β (200 U/ml) for 8 h and amplified by RT-PCR using IL-1β primers specific for amplification of endogenous HuIL-1β. This ensured selective amplification of IL-1β RNA in the presence of IL-1β AS RNA. Total RNA from the promonocytic HL-60 cell line stimulated with 10 ng/ml PMA for 6 h before RNA extraction served as a positive control for IL-1β expression. The L88/5 HuIL-1β AS transfectants expressed much lower levels of endogenous IL-1β RNA compared with the L88/5 NS control or the L88/5 parental cells (Fig. 3A), whereas all cell lines exhibited comparable levels of β-actin RNA (Fig. 3B). Moreover, these low

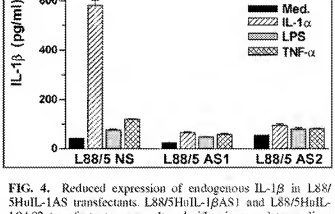

FIG. 4. Reduced expression of endogenous IL-1β in L88/5HuIL-1AS transfectants. L88/5HuIL-1βAS1 and L88/5HuIL-1βAS2 transfectants were cultured either in complete medium alone or in the presence of 50 μg LPS/ml, 500 U TNF-α/ml, or 500 U TNF-α/ml in combination with 100 U IL-1β/ml. As an internal control, cells were also stimulated with 200 U IL-1β/ml. After stimulation for 48 h, the supernatants (SN) were harvested and tested for membrane-bound IL-1β (mIL-1β) using ELISA. To detect mIL-1β, cells were lysed, cell debris was removed by centrifugation, and the remaining lysate was analyzed for IL-1β. Exogenous IL-1β added to the culture was removed during the cell preparations prior to analysis of IL-1β concentrations. The results (mean ± SEM) show representative data from one of four experiments. L88/5 NS, L88/5 cells transfected with a vector containing a nonspecific (nonsense) control DNA sequence; L88/5AS1, L88/5 cells transfected with 136-bp HuIL-1β AS DNA fragment; L88/5AS2, L88/5 cells transfected with 402-bp HuIL-1βAS DNA fragment.

expression levels of IL-1β RNA could be upregulated only slightly on addition of exogenous IL-1β to the culture, suggesting a prominent inhibitory effect of IL-1β AS RNA on endogenous IL-1β RNA processing (Fig. 3A).

Reduced expression of IL-1 protein in stimulated HuIL-1β AS transfectants

The results were confirmed on the protein level of IL-1β cytokine expression. Because we could not detect sufficient amounts of IL-1β in the supernatant, the cells were harvested, cell extracts were prepared, and the membrane-bound/cytosolic form was detected by ELISA. L88/5 HuIL-1βAS transfectants were cultured for 48 h in the presence of various stimuli known to induce IL-1β production, such as lipopolysaccharide (LPS) (50 μg/ml), TNF-α (500 U/ml), and IL-1α (200 U/ml). After stimulation with HuIL-1α, the amounts of IL-1β expressed by the transfectants were significantly reduced by 91% (L88/5AS1) and 86% (L88/5AS2) compared with parental L88/5 cells (data not shown) and L88/5 NS mock control transfectants (Fig. 4). In contrast, LPS and TNF-α failed to elicit a strong overall IL-1β production in controls as well as in L88/5 transfectants.

These results demonstrate the feasibility of directly inhibiting endogenous IL-1β expression using constitutively expressed IL-1β AS RNA.

Expression of HuGM-CSF is impaired in L88/5HuIL-1βAS transfectants and dependent on TNF-α and IL-1β

IL-1β has been described to regulate expression of the hematopoietic growth factors G-CSF, GM-CSF, IL-6, IL-8, and other cytokines in stromal cells.[6–12,29,30] To gain more information on the mechanism(s) of this regulation, we tested the expression of GM-CSF in the L88/5 AS transfectants.

The amounts of GM-CSF detectable in the supernatants of cells cultured in medium only were comparably low in mock-transfected L88/5 cells vs. L88/5 cells expressing HuIL-1β AS RNA (Fig. 5A). However, production of GM-CSF was reduced by 50%–60% in the L88/5 AS transfectants on stimulation with IL-1α (200 U/ml) for 48 h (Fig. 5A). The addition of titrated amounts of exogenous IL-1α confirmed the IL-1β-dependent GM-CSF expression (Fig. 5B). In contrast, synthesis of GM-CSF by L88/5 AS transfectants stimulated with LPS (50 μg/ml)

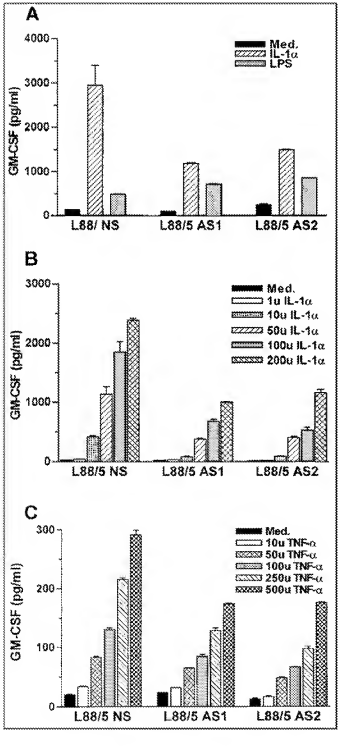

FIG. 5. (**A**) Impaired expression of GM-CSF in L88/5 AS transfectants. L88/5HuIL-1βAS1 and L88/5HuIL-1βAS2 transfectants were cultured either in complete medium alone or in the presence of 50 μg LPS/ml. As an internal control, cells were also stimulated with 200U IL-1α /ml. After stimulation for 48 h, the SN were harvested to be tested for GM-CSF. (**B**) Dose-dependent expression of GM-CSF in L88/5IL-1βAS transfectants after stimulation with IL-1α. L88/5HuIL-1βAS1 and L88/5HuIL-1βAS2 transfectants were maintained in complete medium alone or stimulated with increasing concentrations of IL-1α for 48 h to be analyzed for GM-CSF expression as described in **A**. (**C**) Expression of GM-CSF in L88/5IL-1βAS transfectants after stimulation with TNF-α in the presence of small amounts of endogenous IL-1β. L88/5IL-1βAS1 and L88/5IL-1βAS2 transfectants were stimulated with titrated amounts of TNF-α or maintained in complete medium alone for 48 h. As an internal positive control cells were stimulated with titrated concentrations of IL-1α. After culture, the SN were harvested and tested for GM-CSF. The results in show representative data (mean ± SEM) from one of three experiments. L88/5 NS, L88/5 cells transfected with a vector containing a nonspecific (nonsense) control DNA sequence; L88/5AS1, L88/5 cells transfected with 136-bp HuIL-1β AS DNA fragment; L88/5AS2, L88/5 cells transfected with 402-bp HuIL-1β AS DNA fragment.

was not significantly affected by low amounts of endogenous IL-1β (Fig. 5A).

These data suggested an IL-1β-dependent regulation of GM-CSF expression. As TNF-α has been reported to act synergistically with IL-1α on the production of GM-CSF in stromal cell lines,[12] we examined the effect of TNF-α on the expression of GM-CSF in L88/5 stromal cells. Stimulation of L88/5 stromal cells with TNF-α resulted in an about 6-to 10-fold lower production of GM-CSF compared with stimulation with IL-1α (Fig. 5A,C)(e.g., compare the amounts of about 3000 pg/ml GM-CSF from L88/5 NS control cells after stimulation with IL-1α with about 300 pg/ml GM-CSF obtained after stimulation with TNF-α from the same cells). Stimulation of L88/5 HuIL-1β AS transfectants with titrated amounts of TNF-α for 48 h after detection of GM-CSF in the culture supernatant by ELISA resulted in a reduced production of GM-CSF compared with the L88/5 mock-transfectants(Fig. 5C). This finding demonstrated that GM-CSF expression is decreased in a dose-dependent fashion in the presence of low amounts of endogenous IL-1β.

Production of IL-6 and IL-8 in L88/5 HuIL-1βAS transfectants

To assess whether expression of other cytokines was affected by decreased endogenous IL-1β levels, production of IL-6 and IL-8 was examined. In contrast to the previous findings, synthesis of HuIL-6 and HuIL-8 by L88/5 AS transfectants was not negatively affected after stimulation with such inflammation-associated agents as LPS, TNF-α, and IL-1α (used as positive control) for 48 h (Fig. 6). In fact, production of these two cytokines appeared elevated compared with L88/5 control cells (L88/5 NS).

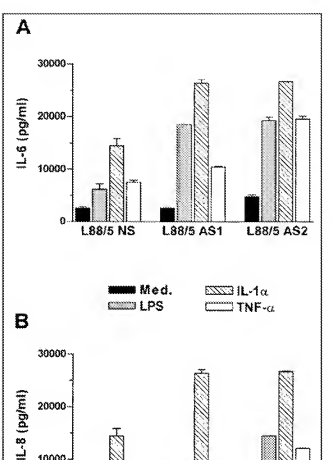

FIG. 6. Production of HuIL-6 (**A**) and HuIL-8 (**B**) in L88/5IL-1AS1 and L88/5IL-1AS2 transfectants after stimulation with IL-1α, LPS, and TNF-α. Stromal cell cultures were stimulated with 50 μg LPS/ml or 500 U TNF-α/ml for 48 h. As an internal control, cells were also stimulated with 200 U IL-1α/ml. SN were tested for IL-6 and IL-8 by ELISA, and the data depicted show duplicate values (mean ± SEM) from one of three experiments. L88/5 NS, L88/5 cells transfected with a vector containing a nonspecific (nonsense) control DNA sequence; L88/AS1, L88/5 cells transfected with 136-bp HuIL-1β AS DNA fragment; L88/5AS2, L88/5 cells transfected with 402-bp HuIL-1βAS DNA fragment.

DISCUSSION

IL-1β has been shown to be a central regulator cytokine in normal and malignant hematopoiesis, suggesting that manipulation of IL-1β expression in stromal cells might be an interesting approach to influence the bone marrow cytokine network. IL-1 exerts its regulatory function not only in hematopoiesis but also in nearly all stress situations challenging the organism in health and disease.[11] Therefore, it seems to be an ideal target to be manipulated *in vitro* under defined conditions, ideally provided by an established and well-characterized cell line.[24,25]

Because of the lack of gene-targeting technologies in human stem cells, modification of IL-1β gene expression in human cells cannot be achieved as it has been possible in the murine system. Therefore, we took advantage of the fact that gene expression can be modified by exogenous nucleic acids. It has been shown that single-stranded DNA (ssDNA) inhibits translation of complementary RNA in a cell-free system,[31] and the existence of naturally occurring AS RNA has been demonstrated in prokaryotes[32] and eukaryotes.[33] These AS molecules regulate the expression of their corresponding genes at the translational level. Because naturally occurring AS RNA seem to be a ubiquitous natural mechanism to regulate gene expression, employment of constitutively expressed AS RNA molecules appears to be an attractive approach to manipulate the expression of targeted genes.

In the present study, human stromal cells were manipulated to express IL-1β AS RNA in a constitutive fashion, with the goal of downregulating endogenous IL-1β expression, thereby also influencing the production of cytokines regulated by IL-1β. The stable expression of AS RNA using gene transfer technologies was chosen to achieve constant intracellular AS effects for a prolonged period of time, which would not have been guaranteed with exogenous AS RNA treatment of the cell cultures.

Two stable transfected cell lines expressing IL-1β AS RNA, L88/5AS1 and L88/5AS2, were generated (Figs. 2 and 3) and

further characterized with regard to changes in endogenous cytokine expression and cytokine production following various exogenous stimuli. In contrast to control transfectants, the level of endogenous IL-1β RNA expression was strongly reduced in these AS transfectants, as measured by semiquantitative RT-PCR (Fig. 3). These results demonstrate the possibility of directly inhibiting IL-1β expression using this AS strategy. Despite different RNA levels, the amounts of membrane-bound and cytosolic IL-1β protein expressed in nonstimulated AS transfectants and mock controls appeared similar, each ranging in the low concentrations (Fig. 4). Immunodetection methods for measuring IL-1β detect primarily the mature form of IL-1β, a 17-kDa molecule, whereas the precursor ProIL-1β is not recognized by commercially available capture antibodies, such as that used in our studies.[34] This may account for the differences in RNA level, but not in protein level, detected in nonstimulated cells (Fig. 4). Interestingly, the IL-1β precursor molecule ProIL-1β lacks a signal sequence known for other cytokines to direct its extracellular transport. Instead, it is cleaved by intracellular cysteine proteases (ICE) to its active form after externalization.[35]

IL-1β is a cytokine that plays its most important physiologic role when an organism is challenged by disease. For example, it has been shown that levels of IL-1 and its specific antagonist IL-1RA are drastically elevated in myocardial infarction and infection.[3] Therefore, potent stimulators of IL-1β expression in stromal cells, such as LPS, IL-1α, and TNF-α, in adequate concentrations were added. Stimulation with each of these molecules could only partially overcome the inhibitory effect of the endogenously produced AS RNA. This inhibitory AS effect was most apparent after stimulation with IL-1α, which caused a massive induction of IL-1β in control transfectants, whereas AS transfectants showed a moderate increase in IL-1β production (Fig. 4). These results demonstrate that there is a specific and potent effect of endogenously expressed AS RNA on the endogenous expression of IL-1β.

One main interest of the present study was to investigate the role of IL-1β on production of hematopoietic growth factors by bone marrow stromal cells. IL-1α and TNF-α synergistically increase G-CSF and GM-CSF production of human marrow stromal cells.[12] The results shown in Figures 5A and C demonstrate that GM-CSF production is strongly reduced in L88/5 transfectants in the presence of IL-1β AS RNA on stimulation with TNF-α and IL-1α. Moreover, this inhibition of GM-CSF expression in L88/5AS transfectants appears to be dose dependent after the addition of titrated amounts of exogenous IL-1α (Fig. 5B). This dose dependence at a much lower GM-CSF protein level could be confirmed by adding increasing amounts of TNF-α to stromal cell cultures (Fig. 5C).

In contrast to decreased GM-CSF production in IL-1β AS transfectants, production of IL-6 and IL-8 was elevated compared with mock controls, especially when potent stimulators of IL-6 and IL-8 production were added (Fig. 6). These findings suggest a negative regulatory effect of IL-1β in L88/5 cells, although previous investigations have reported stimulation of IL-6 and IL-8 production by IL-1β in cultures of primary normal human marrow stromal cells[30] and murine bone marrow cells.[29] Our results might reflect the use of different *in vitro* systems. Current studies are in progress to analyze this effect in more detail. In particular, the role of such inhibitory endogenous cytokines as transforming growth factor-β (TGF-β) and IL-1RA will be evaluated. The elevation of IL-6 and IL-8 secretion in AS RNA transfectants shows that expression of AS RNA is not generally associated with impaired cytokine production and reveals a differential regulatory function of IL-1β in the marrow cytokine network.

Increased IL-1β production has been reported in patients with solid tumors and leukemias.[9] Acute and chronic myeloid leukemia (AML, CML) are the hematologic disorders with the most consistent spontaneous IL-1β gene activation and production.[36–38] As IL-1 is a potent inducer of myeloid growth factors in bone marrow cells,[17] one mechanism of IL-1 action in CML might be the stimulation of stromal cell growth factor production by CML cells themselves. Coculturing bcr/abl-positive CML blast crisis cells onto preestablished adherent layers induced the expression of IL-1β and IL-6 genes, which seem to play a role in disease progression.[38,39] In addition to this indirect effect mediated by stromal cells, IL-1 appears to directly increase hematopoietic progenitor cell proliferation and differentiation in costimulation with growth factors.[5] Cocultures of bcr/abl-positive cells on the IL-1β AS RNA-expressing stromal cell lines generated in the present study could give further insight into the role of IL-1 in autocrine and paracrine mechanisms in CML. We have evaluated parental and transfected L88/5 stromal cells for long term support of human hematopoiesis, but unfortunately because of insufficient long-term feeder capacity for normal and malignant progenitor cells in both L88/5 parental and transfected stromal layers, such questions could not be evaluated (data not shown).

The present study demonstrates that IL-1β plays an important role in regulating the cytokine production of bone marrow stromal cells. Employment of IL-1β AS constructs conferring constitutive expression of IL-1β AS RNA is a potent tool to inhibit endogenous IL-1β production. The production of stromal cell-derived GM-CSF is IL-1β dependent and can be enhanced by exogenous IL-1α and TNF-α in a dose-dependent fashion. In contrast to GM-CSF, the production of IL-6 and IL-8 by IL-1β AS transfected stromal cells is increased, suggesting a negative regulatory effect of IL-1β in L88/5 cells and showing the functional integrity of AS-expressing stromal cells. IL-1β AS RNA expression in stromal cell lines that support human hematopoiesis more efficiently than L88/5 will allow characterization of the role of endogenous IL-1β in malignant hematopoiesis.

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